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Why Does the Growth Cone Cross the Road?

Minireview

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For the great majority of animals—certainly those of neurobiological interest—the body is bilateral, comprising a left side and a right side, which are near-mirror images across the plane of the midline. Since the nervous system is accordingly also bilateral, information must be transmitted from one side to the other in order for the organism to integrate sensory input and coordinate its movement. This requires some neurons to project to and then across the midline. Multiple decussation (projection across the midline) is very rare: axons usually cross the midline either one or zero times. In the vertebrate spinal cord and invertebrate ventral nerve cord, at least some of the molecules that are responsible for attracting growing axons to the midline are now known. Diffusible molecules from the UNC-6/netrin family form a gradient emanating from the midline, which is then detected by receptors from the UNC-40/DCC/frazzled family (for review, see Tessier-Lavigne and Goodman, 1996).

Less is known about the molecules that come into play once growing axons reach the midline. The axons' behavior depends on cell type (Figure 1). Nondecussating axons project longitudinally next to the midline for significant distances without ever crossing. Decussating axons, on the other hand, cross the midline and then somehow remember having done so, projecting further without crossing back, often while staying in close proximity to the midline. The act of crossing seems to transform the behavior of a decussating axon into that of a nondecussating axon. The molecular basis of this change in behavior has been proposed to be regionally restricted expression of cell surface molecules: for instance, rat spinal commissural axons express TAG-1 until they cross the midline and then L1 afterward (Dodd et al., 1988). However, for chick spinal commissural axons, axonin-1 (the homolog of TAG-1) and NgCAM (the homolog of L1) appear to be expressed uniformly along the length of the axons, although their expression is temporally regulated (cf. Stoeckli et al., 1997). Despite this lack of an obvious molecular change at the midline, the chick commissural projection has been one of the systems in which midline crossing is best-understood at the molecular level. The molecule NrCAM, a member of the immunoglobulin superfamily expressed specifically on the midline floor plate, seems to be the signal for commissural axons to cross. Antibody blocking experiments in vitro show that the midline possesses an activity that repulses commissural axons and that an attractive interaction between axonin-1 (on the axons) and NrCAM (on the floor plate) is necessary to overcome this repulsion (Stoeckli et al., 1997).

Genetic Screens for Midline Crossing

Genetic screens, especially in the fly, are starting to provide further insights into the mechanisms of midline crossing. Several years ago, a screen for mutations affecting the ventral nerve cord of *Drosophila melanogaster*, assayed by labeling all CNS axons with the BP102 monoclonal antibody, found two genes that have striking effects on decussation (Figure 2A; Seeger et al., 1993). *commissureless* (*comm*) mutants show reduction or total loss of commissures, while in *roundabout* (*robo*) mutants, the nerve cord resembles a series of automobile roundabouts (thickened commissure pairs) connected by severely reduced longitudinal fascicles. Antibodies that label different subsets of axons can be used to look at errors in their initial outgrowth and reveal that these errors correlate with the global defects in commissures and longitudinals. In *comm*, decussating axons such as SP1 fail to cross the midline, projecting instead directly into the PNS (Figure 2A; Tear et al., 1996). In *robo*, nondecussating axons such as vMP2 and pCC cross the midline abnormally, while decussating axons such as SP1 can be seen to cross the midline more than once (Figure 2A; Kidd et al., 1998a). As the ventral nerve cord develops, it rapidly becomes extremely crowded, making it difficult to follow single axons over a long period. The *apterous-tau-lacZ* transgene is therefore very useful: it labels a very small subset of neurons (the Ap cells) and their axons, showing that these normally nondecussating axons can cross the midline multiple times in *robo* homozygotes (Figure 2A) and occasionally even in heterozygotes (Kidd et al., 1998a). *comm*; *robo* double mutants exhibit the *robo* phenotype, suggesting that *comm* and *robo* act in the same signaling pathway, with *robo* downstream of *comm* (Seeger et al., 1993).

Other genes that affect the overall morphology of the *Drosophila* ventral nerve cord are *abl*, the gene for the Abelson cytoplasmic tyrosine kinase, and three genes that interact with it genetically: *fasciclin I* (*fas I*), *disabled* (*dab*), and *enabled* (*ena*). Abl protein is expressed at high levels in both commissural and longitudinal axons during development. The Fas I protein is a cell adhesion molecule expressed mostly on commissural axons, while Dab is a cytoplasmic protein expressed in axons that is a presumptive substrate for Abl (Gertler et al., 1993). *abl* single mutants do not have significant embryonic CNS defects, but *abl*; *fas I* double mutants have defects, especially a lack of commissures (Figure 2B; Elkins et al., 1990), and *abl*; *dab* double mutants have defects in the commissures and longitudinals (Figure 2B; Gertler et al., 1993). This suggests that *fas I* and *dab* act in pathways that are genetically redundant with *abl*. While the *dab* gene was originally found by screening for genes that enhance *abl*, *enabled* was found by screening for genes that suppress *abl*. Ena is a cytoplasmic protein expressed in axons that contains several proline-rich domains and is phosphorylated by Abl. *ena* homozygotes show variable defects in the CNS,

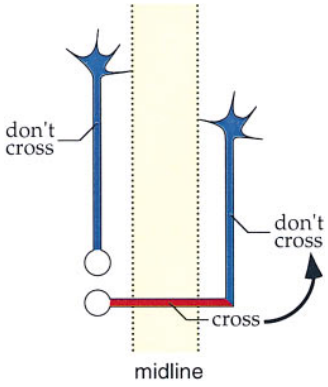

Figure 1. Growth Cone Behavior at the Midline

Normal behavior of decussating and nondecussating axons at the midline. The colors indicate growth-cone behavior: crossing (red) and noncrossing (blue).

including some embryos with a *robo*-like phenotype (Figure 2B; Gertler et al., 1995). The behavior of individual axons in these various mutants will be crucial for testing whether their gross CNS phenotypes reflect specific defects in axon guidance or merely nonspecific effects on axon growth. In *abl*; *fas I* double mutants, staining with the 2D5 antibody shows that the RP1 axon does not cross the midline but instead projects appropriately on the ipsilateral side (Elkins et al., 1990). Further experiments with specific reagents should reveal whether *abl*, *dab*, *ena*, and *fas I* act to control midline crossing in general.

Midline crossing mutants have recently been identified in other species. A *Caenorhabditis elegans* screen for nerve ring defects has been performed using a transgenic line that expresses GFP in a subset of neurons. One of the resulting genes, *sax-3*, controls midline crossing as well as ventral-directed axon navigation (Zallen et al., 1998). Although the worm nervous system is asymmetric, it is still bilateral (Figure 3), and its simplicity makes it possible to follow individual axons using either anti-serotonin antibodies or GFP transgenes. In *sax-3* (for sensory *ax*on defect) mutants, axons from several identified neurons fail to reach the ventral nerve cord, cross the midline aberrantly, or cross more than once (Figure 3; Zallen et al., 1998). Finally, a large-scale zebrafish screen has found mutants defective in left/right motor coordination (Granato et al., 1996), some of which may prove to be defects in midline guidance, as well as mutants in which retinotectal axons either fail to cross the midline or cross more than once (Karlstrom et al., 1996).

Cloning of comm, robo, *and* sax-3

Several years ago, the *comm* gene was cloned and an antibody generated (Tear et al., 1996). Comm is a membrane protein without significant similarity to known molecules. No homolog has been detected in the ~80% of the *C. elegans* genome sequenced so far, nor has a vertebrate homolog been found despite extensive efforts by several labs. *Comm* mRNA is expressed in midline glial cells, which puts it in the right place and time to signal axons approaching the midline; surprisingly, the protein is found not only in the midline glial cells but

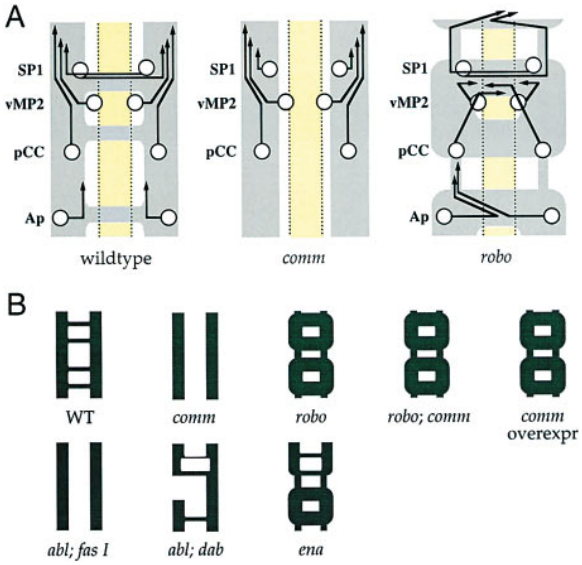

Figure 2. Mutations Affecting the Fly Ventral Nerve Cord

(A) Schematic dorsal views showing wild-type, *comm*, and *robo* homozygote embryos. The commissures and longitudinals are shown as gray in the background, with characteristic paths taken by a subset of identified axons shown in the foreground. (The Ap axons in *comm* have not yet been characterized.)

(B) Summary of different genotypes affecting ventral nerve cord formation. *comm; robo* double mutants and *comm* overexpression mutants phenocopy *robo*, while *abl; fas I* double mutants resemble *comm*. *abl; dab* double mutants affect both commissures and longitudinals, and in *ena* mutants, commissures are thickened at the expense of longitudinals.

also in commissural axons. This suggested that these axons pick up and internalize Comm as they cross the midline glial cells, perhaps as part of a signaling mechanism, but left open several questions, especially those pertaining to the interaction with *robo*.

robo and *sax-3* have now been molecularly cloned, as described in two current papers in *Cell* (Kidd et al., 1998b; Zallen et al., 1998). They are revealed to have

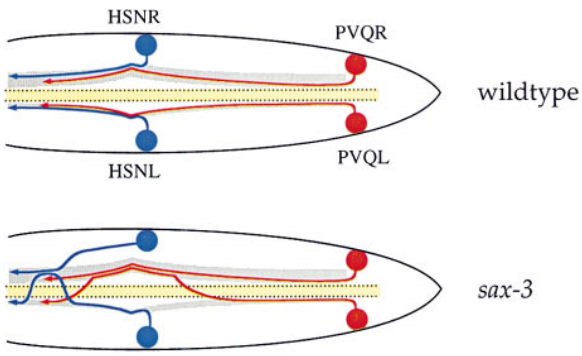

Figure 3. sax-3 Affects Midline Guidance in *C. elegans*

Schematic dorsal views showing the highly asymmetric ventral nervous system of *C. elegans*, with the right nerve cord much larger than the left. In *sax-3* individuals, neurons from the identified pairs HSN and PVQ cross the midline aberrantly. Some axons also make mistakes as they pathfind ventrally to the nerve cord (e.g., the route shown for HSNR in the mutant).

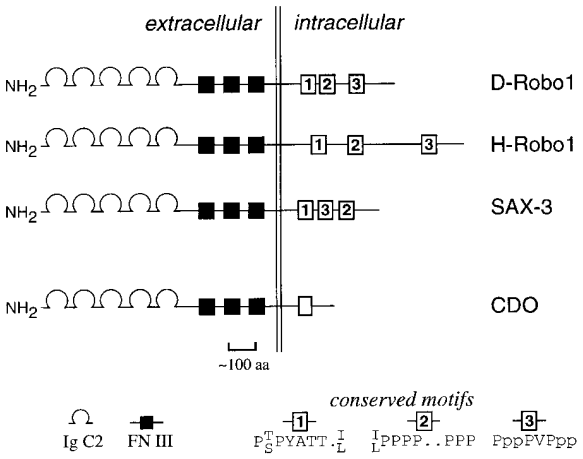

Figure 4. Structure of Robo Family Members

Comparison of Robo family members: *Drosophila* Robo1 (D-Robo1), human Robo1 (H-Robo1); *C. elegans* SAX-3; and CDO, the only other known gene with five IgC2 (immunoglobulin type C2) and three FN III (fibronectin type III) repeats. D-Robo2, H-Robo2, and rat Robo1 (not shown) have very similar structures. Boxed numbers show conserved motifs: motif 1 is a potential tyrosine phosphorylation site, while 2 and 3 are proline-rich motifs that may serve as protein binding sites. In the consensus sequences, the residues shown are those that are identical or conservatively changed among D-Robo1, H-Robo1, and SAX-3, except for p, which represents a proline present in two of the three sequences. The single proline-rich motif of CDO is substantially divergent from Robo motifs 2 and 3.

similar structures and are likely to be homologs of each other (Figure 4). Both are transmembrane molecules with an extracellular domain comprising five immunoglobulin repeats and three fibronectin repeats and an intracellular domain with no obvious catalytic functions. This 5 + 3 domain structure is so far rare; the only previously described protein with this structure is rat CDO (from *C*AM-related/*d*own-regulated by *onc*ogenes), a cell surface glycoprotein isolated from a transformation-resistant cell line (Kang et al., 1997). Together with CDO, Robo and SAX-3 thus form a new subfamily of the immunoglobulin superfamily. Searches of the databases and cDNA libraries yielded four more family members: a second fly gene, *D-Robo2*; a rat gene, *R-Robo1*; and two human genes, *H-robo1/DUTT1* and *H-robo2* (Kidd et al., 1998b). Comparison of the intracellular domains revealed three conserved motifs: one potential tyrosine phosphorylation site and two proline-rich domains that are potential protein binding sites (Figure 4; Kidd et al., 1998b). Intriguingly, one of the proline-rich domains contains the sequence LPPPP, which matches a peptide that is bound by the mouse Ena homolog (Niebuhr et al., 1997). The structures of D-Robo1 and Sax-3 are thus consistent with a function as axon guidance receptors that signal through proteins bound to their intracellular domains.

Complementary expression and functional studies of D-Robo1 and Sax-3 convincingly implicate them as axon guidance receptors (Kidd et al., 1998b; Zallen et al., 1998). An antibody against Robo and a *sax3::GFP* fusion transgene show that they are expressed in the right neurons at the right times to control midline guidance. (Both also show ectodermal and muscle expression, whose function is as yet unknown.) Robo protein is expressed at high levels on longitudinal tracts and low levels on commissures and cell bodies. Immuno-EM shows that Robo is particularly abundant on growth cones and filopodia, and using serial EM sections, it could be seen that a single axon expressed low levels of Robo while in a commissure and then high levels once it turned onto a longitudinal tract (Kidd et al., 1998b). Transgenic rescue of the original mutant phenotypes confirms that the correct genes have been cloned. Furthermore, expression of Robo driven by the ftz_{ng}*-GAL4* element, specific to a subset of CNS neurons, is sufficient to rescue the axons of several identified neurons. Temperature-shift experiments with a temperature-sensitive allele of *sax-3* show that SAX-3 is required at roughly the time that axons are growing out. Thus, the transgenes are only required where and when the axons are growing near the midline. In summary, both the expression and function of Robo and SAX-3 suggest that they act as receptors for a signal that prevents nondecussating axons from crossing the midline and prevents decussating axons from crossing back across the midline. The signal could be either an attractive molecule on the longitudinal axons that keeps other axons fasciculated with them or a midline repellent that inhibits axons from crossing. There are several pieces of circumstantial evidence for the midline repellent hypothesis, including the lack of midline-crossing defects in other mutants that affect axon fasciculation (Kidd et al., 1998b; Zallen et al., 1998). However, a definitive answer to this question awaits molecular characterization of the Robo ligand and determination of its distribution.

What regulates the spatial distribution of Robo? Surprisingly, the CNS looks wild-type in embryos in which Robo is overexpressed. Staining with the Robo antibody reveals why: Robo is present at high levels, but its spatial distribution is so tightly regulated that there is still very little protein on the commissures. From the previous genetic evidence (the *comm*; *robo* double mutant phenotype), *comm* was an obvious candidate to regulate Robo distribution, and this is confirmed by the results of misexpression experiments. In some *comm* hypomorphs, there is more Robo on the commissures than usual, and when Comm is overexpressed pan-neurally, Robo is drastically down-regulated everywhere, and a *robo*-like phenotype results (Kidd et al., 1998a). The simplest model is that Comm *locally* regulates Robo levels (perhaps by causing Robo to be cointernalized?), thus regulating its function. Comm from the midline glia could locally down-regulate Robo on decussating axons to let them cross the midline; once they crossed, Robo levels would rise again and keep the axons from recrossing. So far it has been difficult to reexpress Comm locally at the midline (Kidd et al., 1998a), but this should be possible using FLP constructs to generate mosaics. One can imagine testing this hypothesis at the single-cell level by, for instance, reexpressing Comm in a few midline glia and an epitope-tagged Robo in a few neurons in a *comm* background, and seeing if Robo levels were only reduced at points of apposition to the Comm-expressing cells.

Future Directions

What functions do the other Robo family members play? The expression pattern of *R-Robo1* mRNA in the spinal cord is consistent with expression in the commissural neurons (as well as other cells; Kidd et al., 1998a). The obvious hypothesis is that R-Robo1 acts as a crossing signal receptor for the commissural axons. Its activity might be modulated by an R-Comm, but it is also possible there is no vertebrate Comm homolog and that Comm signaling is replaced in vertebrates by the interaction between axonin-1 and NrCAM. There is also the question of D-Robo2. In worms, SAX-3 plays two functions, controlling midline crossing but also guiding axons ventrally toward the midline (Zallen et al., 1998). It is conceivable that these functions have split in the fly, with D-Robo1 controlling midline crossing and D-Robo2 having a role in ventral guidance. It would be interesting to see if the two aspects of the *sax-3* phenotype could be rescued independently by *D-robo1* and *D-robo2* transgenes.

What are the upstream ligand and downstream signals for the Robo receptor? While immunoglobulin superfamily molecules can bind diffusible signals (e.g., DCC and netrin), it seems more likely that the Robo ligand will be a cell surface molecule. None of the mutants from the original screen is an obvious candidate, but perhaps the ligand can be found with a biochemical approach. On the downstream side, there are obvious candidates for signaling partners. Given both the known CNS phenotypes and the intracellular motifs conserved among the Robo homologs, it is likely that *abl*, *dab*, and *ena* will play roles in Robo signaling.

Finally, the cell biology of Robo signaling promises to be fascinating. How does Robo control the direction of the growth cone? How is Comm taken up by the commissural axons, and how is Robo really localized? Is this part of a general mechanism for localizing proteins to different axonal domains? Answering these questions will help us to understand not just how the growth cone crosses the midline, but how it remembers that it has done so.

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